ANTHRAX TOXIN: PRIMARY SITE OF ACTION

NORMAN S. REMMELE, FREDERICK KLEIN, JAMES A. VICK, JERRY S. WALKER, BILL G. MAHLANDT AND RALPH E. LINCOLN

FROM THE DEPARTMENT OF THE ARMY, FORT DETRICK, FREDERICK, MARYLAND 21701

Reprinted from the Journal of Infectious Diseases
February, 1968, Vol. 118
Pages 104-113

GEORGE BANTA COMPANY, MENASHA, WIS.

ANTHRAX TOXIN: PRIMARY SITE OF ACTION*†

NORMAN S. REMMELE, FREDERICK KLEIN, JAMES A. VICK,‡ JERRY S. WALKER, BILL G. MAHLANDT AND RALPH E. LINCOLN

From the Department of the Army, Fort Detrick, Frederick, Maryland 21701.

Since Keppie et al (1955) demonstrated that a lethal toxin was liberated during in vivo growth of the *Bacillus anthracis* organism, the site of action and mechanism of death have been the subject of study and some controversy. The principal theories of death advanced by workers since 1955 are those of (a) secondary shock, which the authors (Smith and Keppie, 1955) defined as a pathological state involving circulatory failure; (b) asphyxia, because the extremely low concentration of blood oxygen was noted by Nordberg et al (1961) as the actual cause of death by anthrax; and (c) direct effect on the central nervous system as the primary site of action (Lincoln et al, 1964). This latter hypothesis is strengthened by 2 accompanying papers (Vick et al, 1968; Klein et al, 1968) of this series which show depression of the cortical electrical activity following toxin and spore challenge. In addition, Eckert and Bonventre (1963), working with the toxin only, verified that hypoxia occurred in rats following challenge. The principal action of this phenomenon in the rat was interpreted by Beall and Dalldorf (1966) as increased permeability of the pulmonary vasculature and by Gray and Archer (1967) as inhibition in lung tissue of regeneration of nicotinamide adenine dinucleotide. In this series Vick et al (1968) have shown depression of the central cortical electrical activity and, more specifically, the central respiratory mechanism of monkeys challenged with toxin. Their data do not show any cardiac changes until immediately before death. The study of the pathophysiology of monkeys, chimpanzees, and rabbits (Klein et al, 1966) dying of anthrax or of toxin challenge confirms the terminal hypoxia and a remarkable parallel response between spore and toxin-challenged hosts. However, it fails to demonstrate a kidney shutdown that accompanies "secondary shock."

Considering this background of conflicting hypotheses of death by anthrax, we considered it of interest to define more specifically the primary site of action of anthrax toxin. This study, therefore, was concerned with the physiological responses obtained when the toxin was placed in direct contact with the proposed central nervous system target center and with determining if death could be prevented when the affected physiological systems were supported.

METHODS

Anthrax toxin was prepared and its potency was determined by a method previously described by Haines et al

Received for publication June 10, 1967.

We wish to extend our thanks to Col. William R. Beisel, U. S. Army Medical Unit, Fort Detrick, for his assistance.

* In conducting the research reported herein, the investigators adhered to "Guide for Laboratory Animal Facilities and Care" established by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

† Presented at the Annual Meeting of the American Veterinary Medical Association, Dallas, Texas, July, 1967.

‡ Present address: Neurology Branch, Medical Division, Edgewood Arsenal, Maryland.

(1965). The only exception was that horse serum was not added after centrifugation and that the toxin was lyophilized after filtration. The toxin was then stored at −20 C. Each tube of toxin was reconstituted with sterile saline to the desired concentration. The chemical characteristics of the toxin are described in an accompanying paper (Vick et al. 1968).

Rhesus monkeys (*Macaca mulatta*) weighing 3.0 to 3.5 kg were used for this experiment. Prior to the injection of toxin each animal was anesthetized with pentobarbital sodium (30 mg per kg). The toxin was injected directly into the cerebrospinal fluid via the cisterna magna (alanto-occipital junction). After subdural placement of the needle 1 ml of cerebrospinal fluid was removed and the 1.5 ml of toxin containing 1000 rat units was injected.

Arterial and venous blood pressures were monitored continuously with no. 54 polyethylene tubing connected to a Statham strain gauge and recorded on an E and M Physiograph. An arterial catheter was placed into the left femoral artery, while venous pressure was recorded from a cannula inserted into the saphenous vein and advanced into the inferior vena cava. Cannulae and tubing between the test animal and recording device contained heparinized saline (10 units per ml). Heart rate, electrocardiogram (EKG), and respiration rate also were followed with a pair of needle-tipped electrodes placed subcutaneously into either side of the chest wall, amplified, and recorded on the physiograph. The heart rate was recorded so that as heart rate increased the width of the trace decreased (figures 1, 3, 4, and 5) and vice versa. Cardiac output was monitored by a sine wave Statham R) M-4001 electromagnetic flow meter; the electrical output was continuously recorded by a multichannel Sanborn 550 M (R) polygraph. The ascending aorta was located by surgery, and a flow probe, 7 to 9 mm in diameter, was positioned immediately above the origin of the coronary arteries. The probe leads were exteriorized through the incision. Values were obtained before challenge and diastolic flow was used for determining zero flow.

RESULTS

Physiological effects of the toxin. — When 1000 rat units of anthrax toxin were injected into the cerebrospinal fluid, the monkeys expired at 6 to 10 minutes, which contrasts with the 30 hours required to kill when 10,000 rat units were administered intravenously (Vick et al. 1968).

The pattern of death could be divided into 3 distinct phases on the basis of clinical observation and monitored physiological responses (figure 1). Phase 1 was characterized clinically by tetanic paralysis and complete cessation of respiration, which followed the injection of toxin by a matter of seconds. Marked changes occurred physiologically within 15 to 20 seconds after injection. The tetanic paralysis and severe muscle contractions produced artifactual changes superimposed on the respiratory trace (figure 1). Thus, the respiratory patterns recorded soon after injection of toxin were due to violent and severe muscle contraction and were not true respiratory movements. Also, during the first 15 seconds central venous pressure increased sharply from a control of 1 to 2 mm of Hg to a maximum of 20 to 24 mm of Hg. At the same time arterial pressure rose from 120 to 200 mm of Hg, with some decrease in pulse pressure. Simultaneously, the heart rate decreased from an average of 130 beats per minute. However, this was quickly followed by a return to normal. At approximately 180 seconds after injection

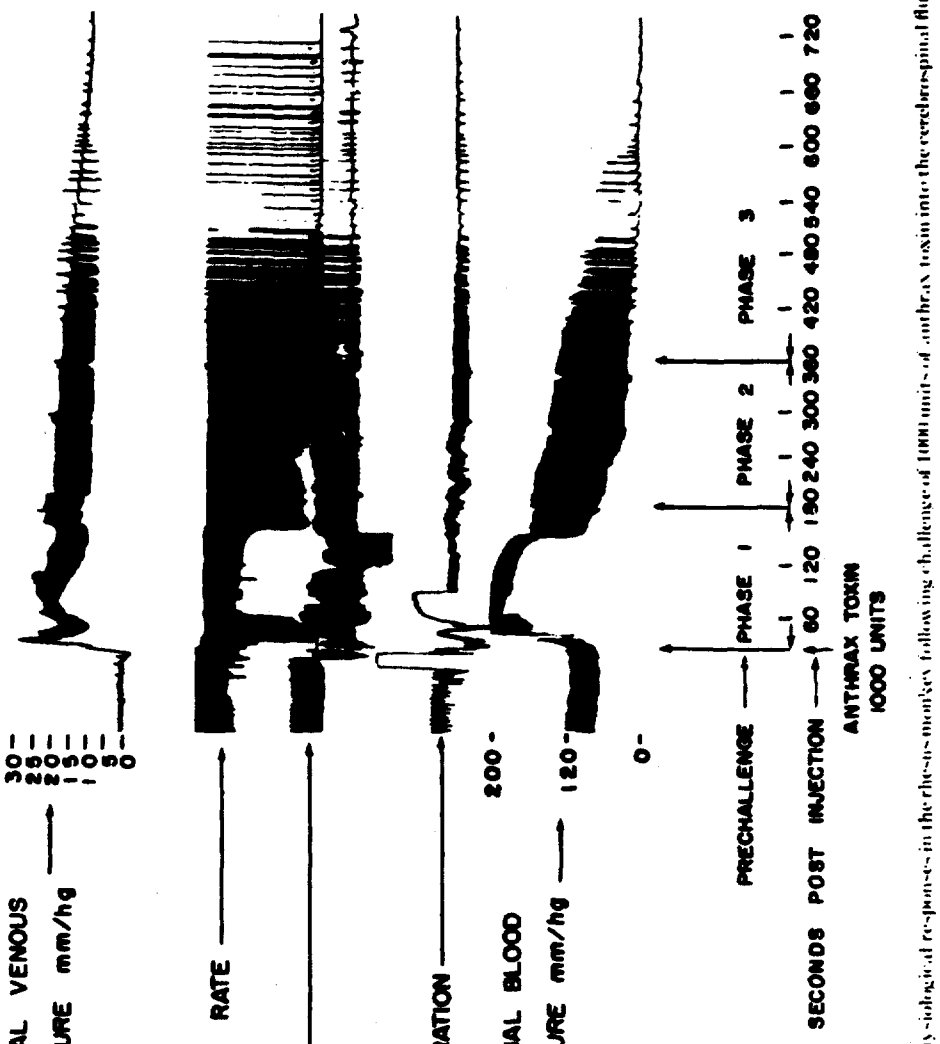

FIGURE 1. Physiological responses in the chest monkey following challenge of 1000 units of anthrax toxin into the cerebrospinal fluid.

changes in EKG were observed (although they are not apparent in figure 1 because of recording techniques). These changes appear consistent with myocardial hypoxia (prolongation of the P-R interval, depression of the T wave, and inversion of the QRS segment).

In the second phase from 180 to 360 seconds, intensive muscle fasciculation developed that was extremely noticeable clinically as well as physiologically. This fasciculation was reflected in the central venous and arterial blood pressure traces (figure 1). The arterial blood pressure began to decrease, with some slowing of the heart rate. The EKG remained abnormal as in the first phase, and some feeble attempts at respiration were made.

Phase 3, the terminal phase from 360 seconds to death, was characterized clinically by a loss of muscle rigidity and generalized flaccidity. Prolonged and progressive hypotension was evident by 360 seconds when the diastolic pressure reached levels of around 25 mm Hg. The pulse pressure was markedly increased and central venous pressure remained above normal until death. The heart rate became irregular and progressively slower, terminating in complete arrest. An increased and inverted T wave was evident from the EKG tracings (figure 1) and again is typical of myocardial hypoxia. Death at 420 to 600 seconds followed embarrassed respiration that began within seconds after injection and continued throughout all 3 phases.

A surgically implanted aortic flow probe and a monitoring of the right and left heart blood pressures revealed the following changes: (a) Within 15 seconds after injection of the toxin into the cerebrospinal fluid, the aortic flow dropped from 780 to 290 ml per minute and at 150 seconds the flow was recorded as 0 (figure 2). (b) The left heart pressure dropped parallel to that of the aorta (c) The right heart pressure showed a sharp rise, as shown by the rise in central venous pressure (figure 1).

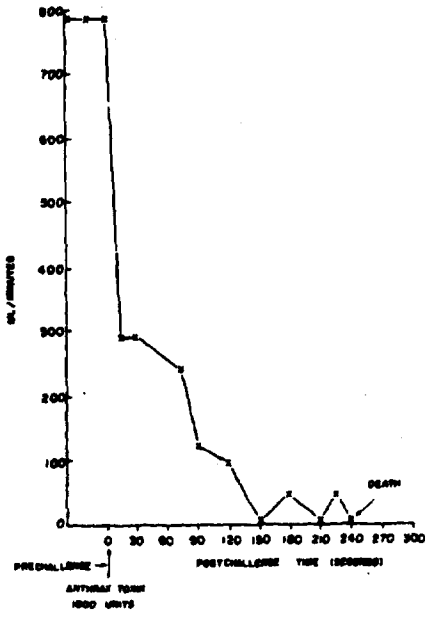

FIGURE 2. Flow rate in the aorta following challenge of 1000 units of anthrax toxin.

Controls. In order to differentiate the action of the toxin from nonspecific responses, several controls were included. For volume and diluent controls 3 monkeys were given subdural injections of sterile saline with volumes of 1, 3 and 5 ml. In addition, 3 ml of cerebrospinal fluid were injected into a 4th monkey to serve as a volume control. For toxin controls inactivated toxin was prepared by 2 different means and injected into several monkeys: (a) 1000 rat units of toxin were reconstituted with antiserum (7000 neutralizing units per ml) in place of the normal saline diluent. This antiserum-neutralized toxin did not kill either rats or monkeys. (b) Toxin (1000 rat units in saline) was boiled in a water bath for 60 minutes, a treatment that has previously been

shown (Vick et al, 1968) to inactivate the lethal activity of the toxin although serological activity remains.

No abnormal physiological or clinical responses were noted with any of the volume or diluent controls. Upon injection of the inactivated toxin (both heat and antiserum-inactivated), the physiological and clinical parameters were similar to those of the regular toxin challenge for the first 2 phases, except that respiration continued very erratically (figure 3). However, after 6 to 8 minutes respiration returned to normal and, although more regular, it was somewhat slower and deeper. The heart rate and EKG also returned toward normal at the same time, as evidenced by the amplitude of the heart rate recording. The continuation of an inverted T wave indicated that myocardial hypoxia still existed. Upon recovery from anesthesia the animals appeared clinically normal and all 4 survived (2 with antiserum-inactivated toxin and 2 with heat-inactivated toxin).

Treatment

Artificial ventilation. From the data presented here and from conclusions drawn from accompanying papers (Vick et al, 1968; Klein et al, 1968) it appeared that, if respiration could be maintained or the smooth muscle contractions overcome (as exemplified by vasoconstriction), then the lethal effects of the toxin could be prevented.

To test the effect of maintaining respiration, animals were placed on a positive-pressure respirator prior to administration of toxin via the cerebrospinal fluid and were maintained in this manner for 10 minutes after injection. Figure 4 shows a reproduction of tracings before and after challenge that are typical of the 5 animals tested. Again, within seconds after toxin challenge the blood pressure increased. It is important to note, however, that the increase in arterial blood pressure was such that the upper limits of the recording galvanometer are exceeded. The EKG became abnormal and the heart rate slower. The tracings for respiration show abnormalities that result from the tremendous muscle spasms that accompany tetanic paralysis and general muscle contraction. Clinically, however, the respiration continued at regular intervals because the animals were on the positive pressure respirator. Note that adequate ventilation was provided to the animals at all times. On the expanded tracings in figure 4 (180 to 192 seconds) irregularities in heart rate and EKG are apparent. These changes were for the most part due to the A-V dissassociation and undoubtedly again to myocardial hypoxia. The blood pressure remained high and did not fall at around 180 seconds, as during phase 2 without maintenance of respiration, as previously noted in figure 1. At 10 minutes the animals were taken off the respirator and were able to maintain their own respiration, as evidenced by the tracings in figure 4 and by visual observation. Blood pressure and heart rate had returned to normal. The EKG still showed some effects of myocardial hypoxia but was greatly improved over that observed at 180 seconds. All 5 animals recovered without any apparent ill effects.

Treatment with isoproterenol. The second approach to overcome the lethal effects of toxin was the use of a beta adrenergic stimulant, DL-isoproterenol hydrochloride (Isoprel, Winthrop Laboratories, New York, New York). Figure 5 shows the tracing obtained from a monkey given 1000 rat units of toxin via the cerebrospinal fluid and then treated with isoproterenol. The clinical and physiological effects were similar, if not identical, to those shown

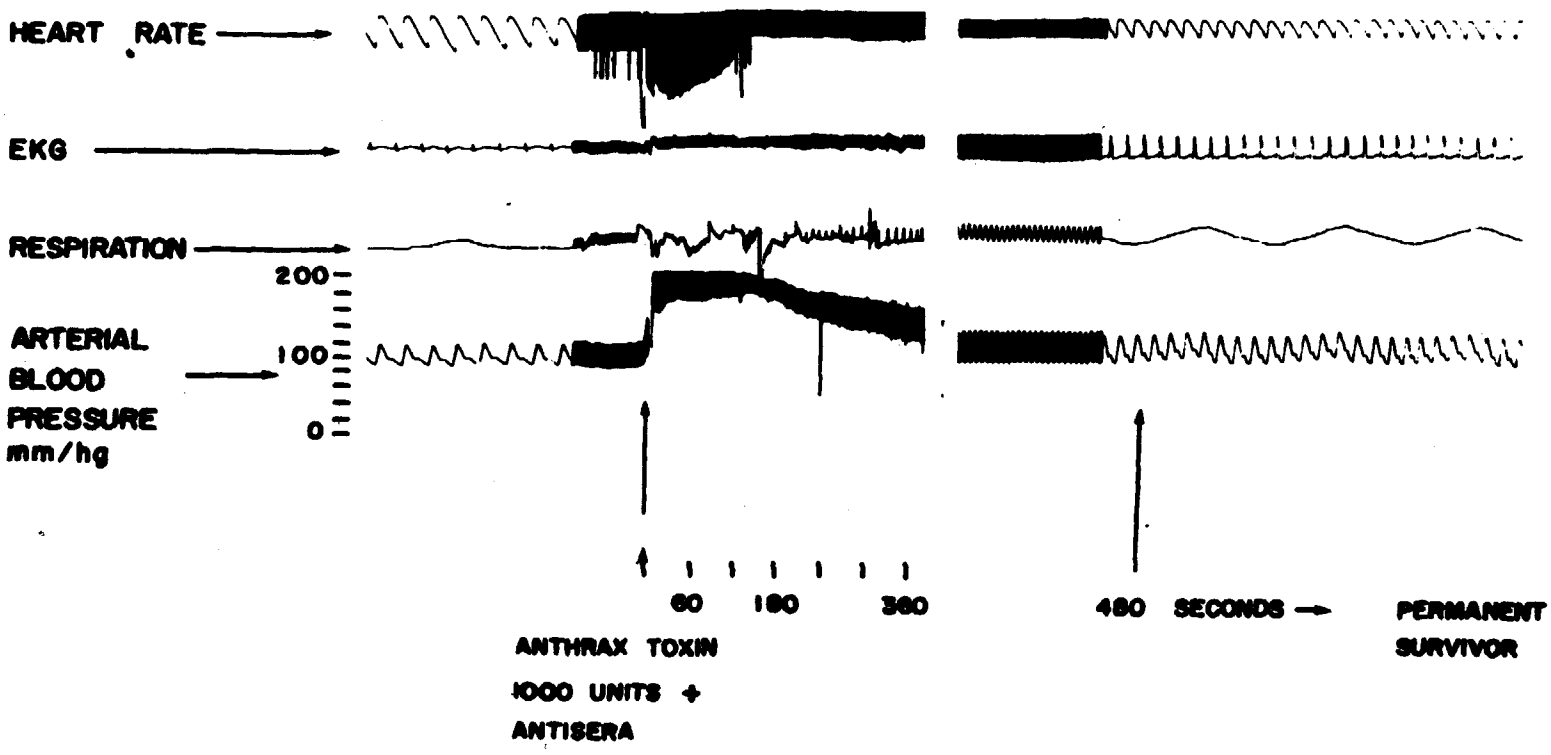

FIGURE 3. Response of a rhesus monkey following challenge of 1000 units of anthrax toxin plus equine antiserum into the cerebrospinal fluid.

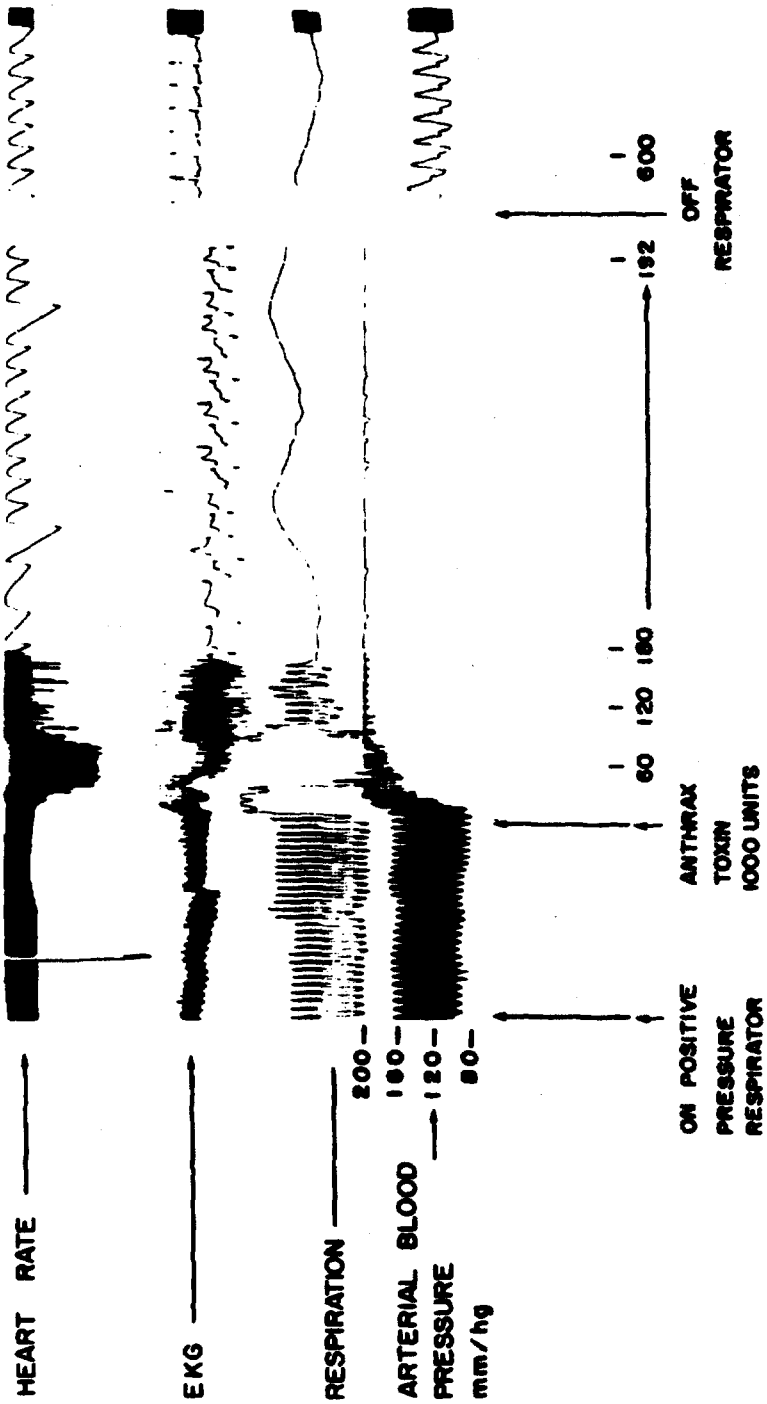

FIGURE 4. Artificial respiratory maintenance following subdural injection of anthrax toxin.

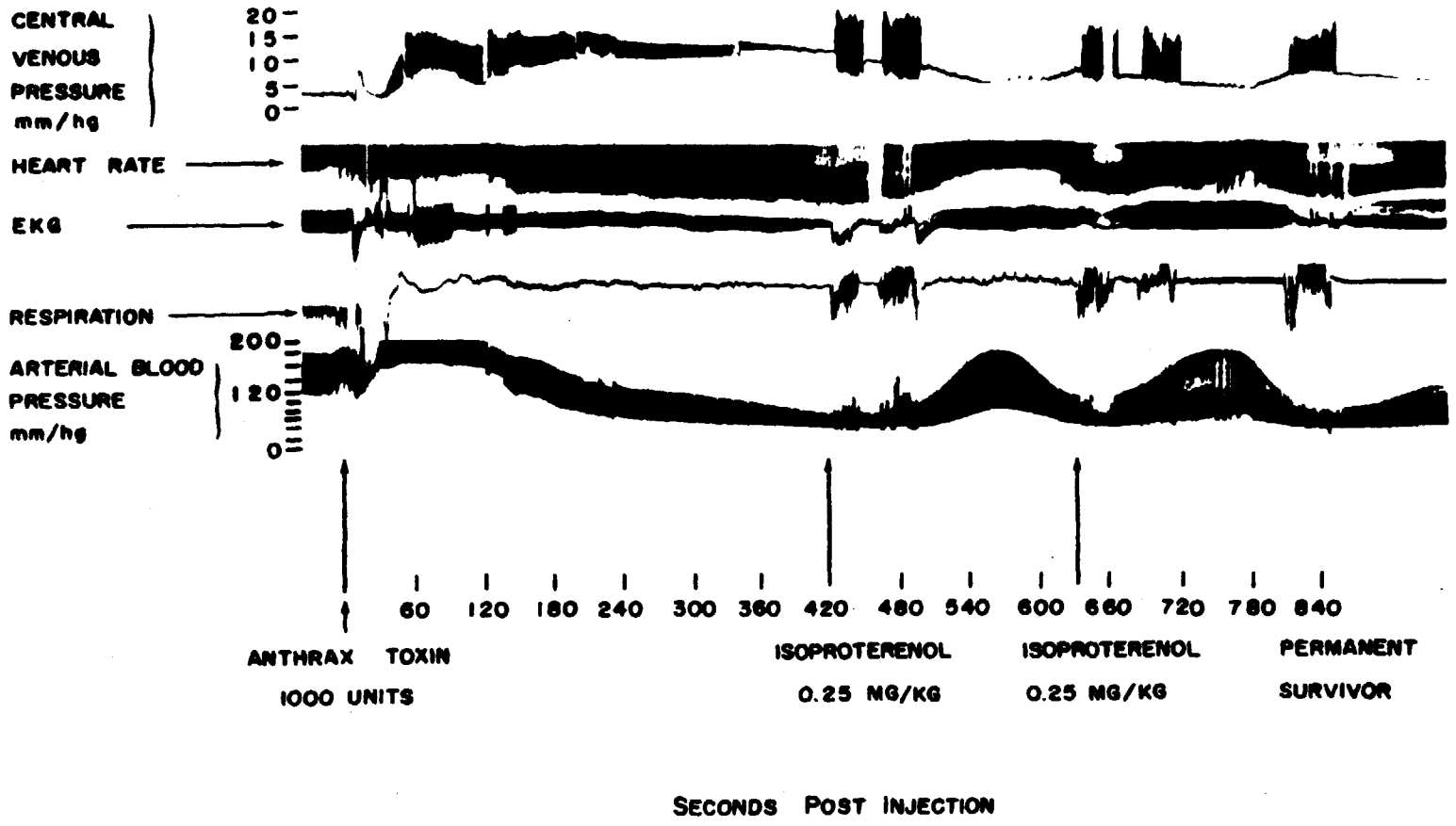

FIGURE 5. Isoproterenol used as supportive therapy following subdural injection of anthrax toxin

in figure 1 until the administration of isoproterenol. At 420 seconds, a time when the blood pressure had reached severe hypotension, i.e., diastolic pressure less than 25 mm of Hg, isoproterenol was slowly administered intravenously at 0.25 mg per kg. Blood pressure increased so that at approximately 540 seconds it was above normal and the central venous pressure had returned toward normal. Heart rate increased. The response of the respiratory system was dramatic in that periods of hyperventilation followed the intravenous infusion of isoproterenol. Following periods of hyperventilation periods of irregular respiration were noted (figure 5). The effect of isoproterenol in respiration was reflected in both the arterial and central venous tracings. These changes are viewed as degree increases in the magnitude of trace and reflect increased intrathoracic pressure. However, by 630 seconds the blood pressure started to fall and a second infusion of isoproterenol administered at the same dose resulted in responses identical to those following the first injection. After 14 minutes the monitored systems tended to normalize themselves. The respiration had become more regular as observed clinically, and all 5 animals treated with isoproterenol survived.

DISCUSSION

Considering the low dosage of toxin required to kill in about 10 minutes and the drastically altered physiology of the host, there is little doubt that anthrax toxin affects the central nervous system. The many changes produced following subdural administration of toxin may be explained by a tremendous increase in central nervous system discharges, which result in severe generalized muscle contraction of both smooth and skeletal muscles, causing first an increase in blood pressure, then a rise in central venous pressure and then a drop in aortic blood flow. The initial abnormalities in EKG may be accounted for the same way. Changes noted in EKG are typical of myocardial hypoxia, which follows the dyspnea noted within 15 seconds after injection.

While it is possible that the toxin may have influenced the central nervous system as a secondary manifestation of a direct cardiovascular effect, evidence provided in the present report (figures 1 and 4) indicates that the central nervous system was, in fact, primary. From our tracings it is evident that respiration was affected virtually instantaneously following injection of toxin, whereas changes in the arterial blood pressure, central venous pressure, heart rate, and EKG followed some 10 to 40 seconds later.

The ultimate effect of the toxin on the central nervous system is anoxia. This is caused by a lack of oxygenation of the blood either by itself or in combination with decreased blood flow. The terminal anoxia could arise from several sources: (a) through a direct effect on the respiratory center in the central nervous system, causing respiratory failure and a lack of oxygenation, (b) through a tetanic paralysis of the intercostal and diaphragmatic muscles, arising from increased central nervous system discharges, again resulting in respiratory paralysis and lack of oxygenation, and (c) through a cardiovascular failure mediated by the increased central nervous system discharges, producing a generalized smooth muscle constriction. The generalized smooth muscle constriction would also cause lack of oxygenation through bronchial constriction.

The results obtained with the inactivated forms of the toxin, i.e., inactivated with antiserum and heat, would suggest that survival depends on respiration continuing because the com-

ponent affecting the respiratory center has been inactivated or the components that cause the central nervous system cardiovascular failure have been altered in such a way that the animal is able to re-establish enough blood flow to prevent terminal anoxia.

Survival following the use of isoproterenol may be attributed to dilatation of the pulmonary vasculature, which would allow uninterrupted flow of blood through the lungs, or to the known action of isoproterenol on the myocardium, which is an increased cardiac output (Best and Taylor, 1961). Either one or both of these effects could maintain or re-establish the function of the central nervous system or especially the respiratory center by increased circulation and oxygenation. The survival obtained by the use of a positive-pressure respirator, however, may be due to one or any combination of the 3 previously mentioned causes of terminal anoxia.

It is a remote possibility that the dramatic changes produced by subdural administration of the toxin are caused by a component of the toxin that cannot cross the blood-brain barrier in the infection or after intravenous challenge. However, we do not feel this is the case. It is more probable that subdural administration accentuates the central nervous system effects.

Except for cases in which specific antiserum was administered, we believe this to be first demonstration that, in animals given lethal amounts of bacterial toxin, death can be prevented by sustaining and maintaining the affected primary physiological system. Whatever the ultimate cause of the central nervous system-mediated anoxia, the therapeutic and supportive treatment of bacterial diseases has now taken on a new and exciting aspect.

SUMMARY

Administration of anthrax toxin via the cerebrospinal fluid demonstrates positively that the toxin alters the body's physiology, primarily via the central nervous system, and kills by terminal anoxia mediated by the central nervous system. The use of a positive-pressure respirator and isoproterenol prevented death in animals that had received lethal doses of toxin. This demonstration of therapy for a lethal bacterial toxin opens new avenues of investigation.

REFERENCES

Beall, F. A. and Dalldorf, F. G. 1966, J Infect Dis 116:377–389.

Best, C. H. and Taylor, N. V. 1961, The physiological basis for medical practice. Baltimore, Williams and Wilkins Company.

Eckert, N. J. and Bonventre, P. F. 1968, J Infect Dis 118: 226–232.

Gray, L. and Archer, L. J. 1967, J Bact 93:36–39

Haines, B. W., Klein, F. and Lincoln, R. E. 1965, J Bact 89:74–83.

Keppie, J., Smith, H. and Harris-Smith, P. W. 1955, Brit J Exp Path 36:315–322.

Klein, F., Lincoln, R. E., Dobbs, J. P., Mahlandt, B. G., Remmele, N. S. and Walker, J. S. 1968, J Infect Dis 118:97–103.

Klein, F., Walker, J. S., Fitzpatrick, D. F., Lincoln, R. E., Mahlandt, B. G., Jones, W. I., Dobbs, J. P. and Hendrix, K. J. 1966, J Infect Dis 116:123–138.

Lincoln, R. E., Walker, J. S., Klein, F. and Haines, B. W. 1964, Adv Vet Sci 9:327–368.

Nordberg, B. K., Schmiterlow, C. G. and Hanson, H. J. 1961, Acta Path Microbiol Scand 53:295–318.

Smith, H. and Keppie, J. 1955, Gen Microbiol 5:126–151.

Vick, J. A., Lincoln, R. E., Klein, F., Mahlandt, B. G., Walker, J. S. and Fish, D. C. 1968, J Infect Dis 118:85–96.